

# Anabolic Effect of Wasabi Leafstalk (*Wasabia japonica* MATSUM.) Extract on Bone Components in the Femoral-Diaphyseal and -Metaphyseal Tissues of Aged Female Rats *in Vitro* and *in Vivo*

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The effect of wasabi leafstalk (*Wasabia japonica* MATSUM.) extract on bone components in the femoral-diaphyseal and -metaphyseal tissues of aged female rats *in vitro* and *in vivo* was investigated. Femoral-diaphyseal and -metaphyseal tissues were cultured for 48 hr in Dulbecco's modified Eagle's medium (serum free) containing either vehicle or wasabi leafstalk extract (10, 25 or 50  $\mu\text{g/ml}$  of medium). The presence of wasabi leafstalk extract (50  $\mu\text{g/ml}$ ) caused a significant increase in calcium content, alkaline phosphatase activity and deoxyribonucleic acid (DNA) content in the diaphyseal and metaphyseal tissues *in vitro*. However, the effect of wasabi leafstalk extract (50  $\mu\text{g/ml}$ ) in increasing bone components was completely abolished in the presence of cycloheximide ( $10^{-6}$  M), an inhibitor of protein synthesis. Moreover, rats were orally administered wasabi leafstalk extract (10 or 20 mg/100 g body weight) once daily for 7 days. The calcium content, alkaline phosphatase activity and DNA content in the femoral-diaphyseal and -metaphyseal tissues of aged rats was significantly increased by the administration of wasabi leafstalk extract (10 or 20 mg/100 g) for 7 days *in vivo*. Meanwhile, body weight, serum calcium and inorganic phosphorus concentrations of female aged rats were not significantly altered by the administration of wasabi leafstalk extract (10 or 20 mg/100 g) for 7 days. The present study demonstrates that wasabi leafstalk extract has an anabolic effect on bone components *in vitro* and *in vivo*. The intake of wasabi leafstalk extract may have a preventive effect on bone loss with increasing age.

**Key words** — bone metabolism, *Wasabia japonica*, wasabi leafstalk, osteoporosis, aging, rat femur

## INTRODUCTION

Bone loss with increasing age induces osteoporosis.<sup>1-3</sup> Osteoporosis is widely recognized as a major public health problem.<sup>4</sup> Bone loss may be due to increased bone resorption and decreased bone formation. A decrease in bone mass leads to bone fracture. The most dramatic expression of osteoporosis can be countered by factors which help to prevent bone loss with increasing age.<sup>5</sup>

Recent studies have shown that isoflavones (including genistein and daidzein), which are contained in large quantities in soybean, have a stimulatory effect on osteoblastic bone formation<sup>6-8</sup> and an in-

hibitory effect on osteoclastic bone resorption,<sup>9-11</sup> thereby increasing bone mass. Also, menaquinone-7, an analogue of vitamin K<sub>2</sub> which is essential for the  $\gamma$ -carboxylation of the osteocalcin of a bone matrix protein, is abundant in fermented soybean. It stimulates osteoblastic bone formation<sup>12</sup> and inhibits osteoclastic bone resorption<sup>13</sup> *in vitro*. Its prolonged dietary preventive effect on bone loss induced by ovariectomy in rats, which is an animal model for osteoporosis, has been demonstrated.<sup>14</sup> Thus, nutritional factors play a role in bone health and may be important in the prevention of bone loss with increasing age in humans.<sup>15-17</sup>

Other food factors have been shown to have an anabolic effect on bone metabolism. More recently, it has been demonstrated that among various marine algae, *Sargassum horneri* (*S. horneri*) has a unique anabolic effect on bone calcification *in vitro* and *in vivo*.<sup>18,19</sup> The anabolic effect of *S. horneri*

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extract on bone components is due to increased bone formation, caused by a newly synthesized protein component, and also to decreased bone resorption.<sup>20,21)</sup>

Of various plant extracts, wasabi leafstalk (*Wasabia japonica* MATSUM.) extract has a unique stimulatory effect on bone calcification *in vitro*.<sup>22,23)</sup> Whether an anabolic effect of wasabi leafstalk extract on bone components is demonstrated in aged female rats, in which bone mass is decreased by aging, has remained unknown. The present study was undertaken to determine the anabolic effect of wasabi leafstalk extract on bone components in aged female rats. We found that wasabi leafstalk extract has an anabolic effect on bone components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of aged female rats *in vitro* and *in vivo*.

## MATERIALS AND METHODS

**Chemicals** — Dulbecco's modified Eagle's medium (MEM) (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (penicillin 5000 U/mg; streptomycin 5000 µg/ml) were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (fraction V) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals, of reagent grade, were from Wako Pure Chemical Industries (Osaka, Japan).

**Wasabi Leafstalk Extracts** — Fresh wasabi leafstalk (*Wasabia japonica* MATSUM.; about 50 g) was homogenized for 3 min in distilled water or 20% ethanol solution (150 ml), and the homogenate was centrifuged at 8000 rpm for 20 min.<sup>22)</sup> The resulting supernatant was filtered through filtration paper, then the filtration solution was extracted 3 times with diethyl ether (about 150 ml). The ether phase was removed, and the resulting aqueous phase was lyophilized. About 0.9 g of the frozen powder was obtained. The powder was dissolved in distilled water. Before its use in bone culture experiments, wasabi leafstalk extract was aseptically filtered through a membrane.

**Animals** — Female Wistar rats (conventional) weighing 200–250 g (50 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and 1.1% phosphorus at a room

temperature of 25°C, with free access to distilled water.

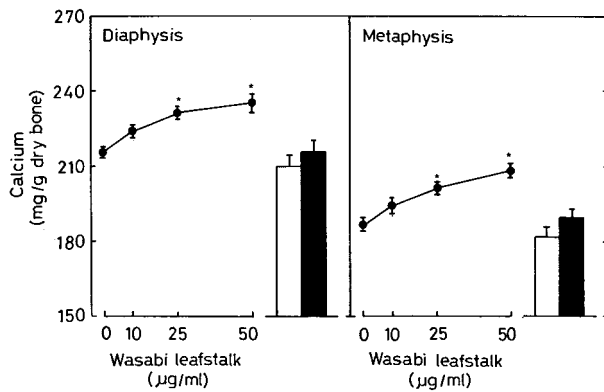
**Bone Culture** — The femurs were removed aseptically after bleeding, and were then soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal and -metaphyseal tissues were cut into small pieces. Diaphyseal or metaphyseal fragments were cultured for 48 hr in a 35 mm dish in 2.0 ml of medium consisting of Dulbecco's MEM (high glucose, 4.5 g/dl) supplemented with 0.25% bovine serum albumin plus antibiotics (penicillin 100 units and streptomycin 100 µg/ml of medium).<sup>24)</sup> In our experiments, bone tissues were cultured in a medium containing either vehicle or Wasabi leafstalk extracts (10, 25 or 50 µg/ml of medium). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO<sub>2</sub> and 95% air.

**Administration Procedures** — The water suspension (10 and 20 mg/ml/100 g body weight) of the powder of an ethanol-solubilized extract of wasabi leafstalk was orally administered to rats through a stomach tube once daily for 7 days. Control rats received distilled water (1.0 ml/100 g body weight) orally. The animals were killed 24 hr after the last administration by cardiac puncture under light ether anesthesia, and the blood and femur were removed immediately.

**Analytical Procedures** — Blood samples were centrifuged for 30 min after collection, and the serum was separated and analyzed immediately. Serum calcium was determined by the method of Willis.<sup>25)</sup> Serum inorganic phosphorus was measured by the method of Taussky and Shon.<sup>26)</sup>

The diaphyseal and metaphyseal tissues were dried for 16 hr at 110°C, weighed, and then dissolved in nitric acid solution. Calcium was determined by atomic absorption spectrophotometry.<sup>24)</sup> The calcium content in bone tissues was expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the metaphyseal tissues were immersed in 3.0 ml of ice-cold barbital buffer 6.6 mM (pH 7.4), cut into small pieces, and disrupted for 60 sec with an ultrasonic device. The supernatant, centrifuged at 600 × *g* for 5 min, was used to measure enzymes. The assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.<sup>27)</sup> Enzyme activity was expressed



**Fig. 1.** Effect of Wasabi Leafstalk Extract on Calcium Content in Rat Femoral-diaphyseal and -metaphyseal Tissues *in Vitro*

Femoral-diaphyseal and -metaphyseal tissues obtained from normal aged rats were cultured for 48 hr in a medium containing either vehicle or wasabi leafstalk extract (0, 10, 25 or 50 µg/ml of medium) in the absence or presence of cycloheximide ( $10^{-6}$  M). Each value is the mean  $\pm$  standard error of the means (S.E.M.) of six rats. \* $p < 0.01$  compared with the control (none) value. White bars, cycloheximide; black bars, wasabi leafstalk extract (50 µg/ml) plus cycloheximide.

as micromol of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentration was determined by the method of Lowry *et al.*<sup>28)</sup>

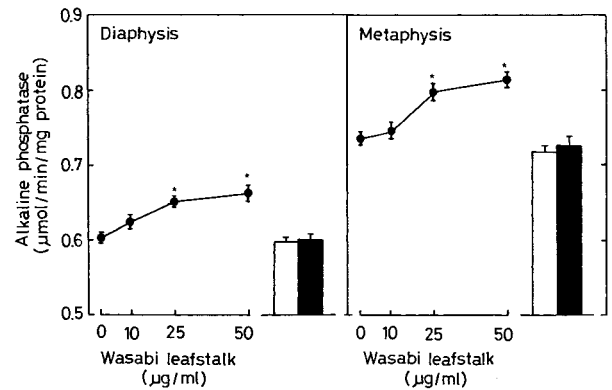
To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 hr after homogenization of the bone tissues.<sup>29)</sup> After alkali extraction, the samples were centrifuged at  $1000 \times g$  for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti<sup>30)</sup> and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

**Statistical Analysis** — The significance of difference between values was estimated by Student's *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

## RESULTS

### Effect of Wasabi Leafstalk Extract on Bone Components *in Vitro*

The effect of wasabi leafstalk extract on calcium content in the femoral-diaphyseal and -metaphyseal tissues obtained from female aged rats *in vitro* is shown in Fig. 1. Bone tissues were cultured for 48 hr in the presence of wasabi leafstalk extract. Diaphyseal and metaphyseal calcium content was significantly increased in the presence of wasabi leafstalk extract (25 or 50 µg/ml), but increasing diaphyseal



**Fig. 2.** Effect of Wasabi Leafstalk Extract on Alkaline Phosphatase Activity in Rat Femoral-diaphyseal and -metaphyseal Tissues *in Vitro*

Femoral-diaphyseal and -metaphyseal tissues obtained from normal aged rats were cultured for 48 hr in a medium containing either vehicle or wasabi leafstalk extract (0, 10, 25 or 50 µg/ml of medium) in the absence or presence of cycloheximide ( $10^{-6}$  M). Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value. White bars, cycloheximide; black bars, wasabi leafstalk extract (50 µg/ml) plus cycloheximide.

and metaphyseal calcium content was completely prevented in the presence of cycloheximide ( $10^{-6}$  M).

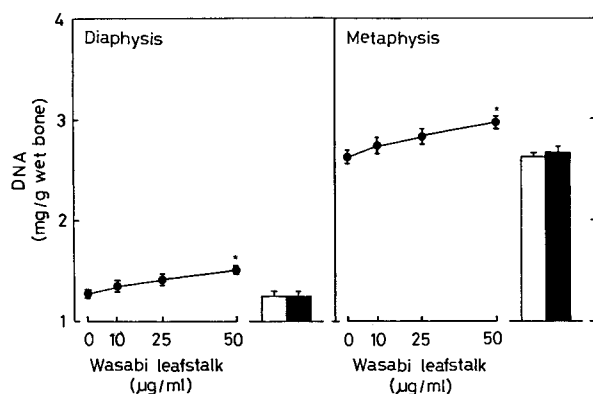
The presence of wasabi leafstalk extract (25 or 50 µg/ml) caused a significant increase in alkaline phosphatase activity in the diaphyseal and metaphyseal tissues *in vitro* (Fig. 2). The stimulatory effect of wasabi leafstalk (50 µg/ml) on diaphyseal and metaphyseal enzyme activity was not seen in the presence of cycloheximide ( $10^{-6}$  M).

Diaphyseal and metaphyseal DNA content was significantly raised in the presence of wasabi leafstalk extract (50 µg/ml) (Fig. 3). This effect was completely prevented in the presence of cycloheximide ( $10^{-6}$  M).

### Effect of Administration of Wasabi Extract on Bone Components in Aged Female Rats *in Vivo*

Body weight, serum calcium and inorganic phosphorus concentrations of aged (50-week-old) rats were not significantly altered by the oral administration of wasabi leafstalk extract (10 or 20 mg/100 g body weight) for 7 days (Table 1).

The oral administration of wasabi leafstalk extract (10 or 20 mg./100 g) to aged female rats caused a significant increase in calcium content (Fig. 4), alkaline phosphatase activity (Fig. 5) and DNA content (Fig. 6) in the femoral-diaphyseal and -metaphyseal tissues *in vivo*.



**Fig. 3.** Effect of Wasabi Leafstalk Extract on DNA Content in Rat Femoral-diaphyseal and -metaphyseal Tissues *in Vitro*

Femoral-diaphyseal and -metaphyseal tissues obtained from normal aged rats were cultured for 48 hr in a medium containing either vehicle or wasabi leafstalk extract (0, 10, 25 or 50  $\mu\text{g/ml}$  of medium) in the absence or presence of cycloheximide ( $10^{-6}$  M). Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value. White bars, cycloheximide; black bars, wasabi leafstalk extract (50  $\mu\text{g/ml}$ ) plus cycloheximide.

## DISCUSSION

Of various plant extracts, wasabi leafstalk (*Wasabia japonica* MATSUM.) extract has been demonstrated to have a unique stimulatory effect on bone calcification *in vitro*.<sup>22)</sup> Characterization of the active component of wasabi leafstalk extract in stimulating bone calcification *in vitro* has been examined.<sup>23)</sup> The components of wasabi leafstalk extract which increase bone calcium content are stable when treated by heating or with acidity or alkalinization.<sup>23)</sup> Thus, the active component of wasabi leafstalk extract thus may not be a protein.

The extract of wasabi leafstalk has been subjected to gel filtration chromatography with a high pressure liquid chromatography system.<sup>23)</sup> From this study, it is assumed that the active component of wasabi leafstalk extract in stimulating bone calcifi-

cation has a longer retention time than that of cytidine (molecular weight; MW. 243) that is used as a standard.<sup>23)</sup> The molecular weight of genistein, which can stimulate bone calcification *in vitro*,<sup>31)</sup> is 270. The active component of wasabi leafstalk may differ from cytidine or genistein. In addition, sinigrin has been shown to have no effect on bone calcification.<sup>23)</sup> Identification of the active component in wasabi leafstalk extract remains to be elucidated.

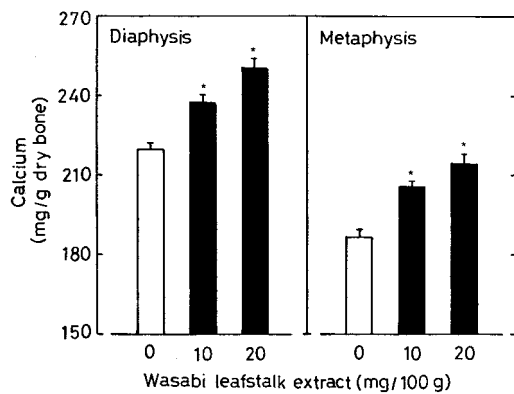
The present study was undertaken to determine whether wasabi leafstalk extract has a preventive effect on bone loss with increasing age, which induces osteoporosis. Bone metabolism in the femoral-diaphyseal and -metaphyseal tissues is deteriorated with increasing age (50 weeks old), as compared with that of young rats (4 weeks old).<sup>19,32)</sup> The presence of wasabi leafstalk extract in culture medium caused a significant increase in calcium content, alkaline phosphatase activity and DNA content in the femoral-diaphyseal and -metaphyseal tissues of aged female rats *in vitro*. The effect of wasabi leafstalk extract in increasing bone components was completely prevented in the presence of cycloheximide, an inhibitor of protein synthesis. This result suggests that an anabolic effect of wasabi leafstalk extract on bone metabolism results from newly synthesized protein components.

Moreover, the oral administration of wasabi leafstalk extract was found to induce a significant increase in calcium content, alkaline phosphatase activity and DNA content in the femoral-diaphyseal and -metaphyseal tissues of aged female rats *in vitro*. Administration of wasabi leafstalk extract did not cause a significant alteration in body weight, serum calcium or inorganic phosphorus concentrations in aged female rats, indicating that the extract may not have a toxic effect. Thus, the intake of wasabi leafstalk extract may have a preventive effect on the deterioration of bone metabolism with increasing age. Presumably, dietary wasabi leafstalk has a role in

**Table 1.** Body Weight, Serum Calcium and Inorganic Phosphorus Concentrations in Female Aged Rats Orally Administered Wasabi Leafstalk Extract

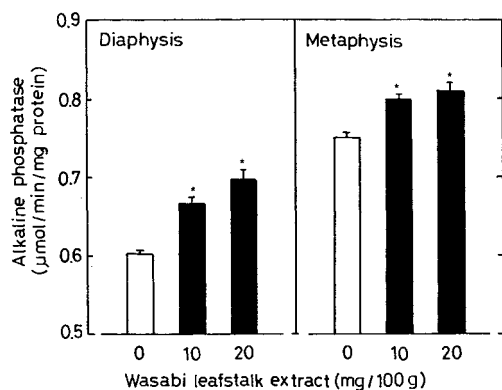
Treatment	Body Weight (g)	Serum concentration (mg/dl)	
		Calcium	Inorganic phosphorus
Control	238.3 $\pm$ 5.23	9.00 $\pm$ 0.11	5.28 $\pm$ 0.18
Wasabi leafstalk extract			
10 mg/100 g	235.3 $\pm$ 4.48	9.01 $\pm$ 0.14	5.24 $\pm$ 0.17
20 mg/100 g	225.3 $\pm$ 4.25	9.02 $\pm$ 0.15	5.30 $\pm$ 0.13

Rats (50 weeks old) were orally administered wasabi leafstalk extract (10 or 20 mg/100 g body weight) for 7 days. Each value is the mean  $\pm$  S.E.M. of six rats. Data were not significant.



**Fig. 4.** Change in Calcium Content in the Femoral-diaphyseal and -metaphyseal Tissues of Aged Rats Orally Administered Wasabi Leafstalk Extract *in Vivo*

Rats were orally administered wasabi leafstalk extract (10 or 20 mg/ml/100 g body weight) once daily for 7 days, and the animals were killed 24 hr after the last administration. Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value.



**Fig. 5.** Change in Alkaline Phosphatase Activity in the Femoral-diaphyseal and -metaphyseal Tissues of Aged rats Orally Administered Wasabi Leafstalk Extract *in Vivo*

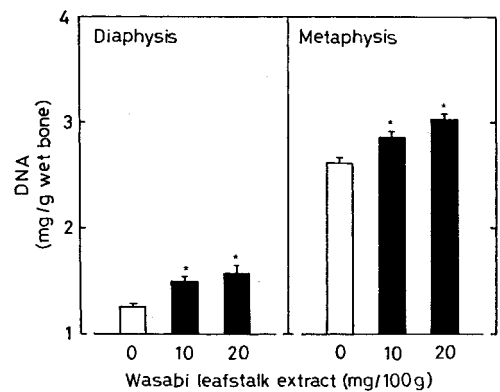
The procedure of administration was described in the legend of Fig. 4. Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value.

the prevention of osteoporosis with aging.

In conclusion, it has been demonstrated that wasabi leafstalk extract has an anabolic effect on bone components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of aged female rats *in vitro* and *in vivo*.

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**Fig. 6.** Change in DNA Content in the Femoral-diaphyseal and -metaphyseal Tissues of Aged rats Orally Administered Wasabi Leafstalk Extract *in Vivo*

The procedure of administration was described in the legend of Fig. 4. Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value.

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